THE ACTIVITY OF PEROXIDASES AND SUPEROXIDE DISMUTASES IN TRANSGENIC PHOSPHINOTHRICIN-RESISTANT *LOTUS CORNICULATUS* SHOOTS

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Abstract - The aim of this study was to investigate the effect of the non-selective herbicide Basta®, with phosphinothricin (PPT) as active compound, on antioxidative enzymes in transgenic PPT-resistant *Lotus corniculatus* cv. Bokor shoots grown under *in vitro* conditions. Analysis of peroxidases (POD) and superoxide dismutases (SOD) showed that the activity of these enzymes was affected by herbicide application more in control PPT-sensitive than in transformed resistant shoots. These results confirmed the capacity of genetically modified resistant shoots to reduce the influence of PPT on the physiological processes and disturbance of oxidative balance in cells.

Key words: Lotus corniculatus, *in vitro* culture, Basta®, phosphinothricin, antioxidative system, peroxidases, SOD

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INTRODUCTION

Basta® (Hoescht A.G., Germany), with its active compound phosphinothricin (PPT), is one of the most common and effective non-selective herbicides used to control weeds, particularly during the establishment of forage grass. Some of its most dangerous effects on plants are the irreversible inhibition of glutamine synthetase and the rapid accumulation of ammonium derived from the activity of nitrate reductase and the photorespiratory pathway which leads to a suppression of photosynthesis, chlorosis, decreased growth (Evstigneeva et al. 2003; Manderscheid and Wild, 1986), inhibition of ribulose-1.5-bisphosphate carboxylase/oxygenase (Rubisco) and consequently CO_2-fixation (Ziegler and Wild, 1989). Similar to other environmental stresses, phosphinothricin also causes a disturbance in redox potential and oxidative damage in plants, either directly or indirectly, by triggering increased levels of reactive oxygen species (ROS) (Qian et al., 2008). An increased production of ROS can have many dangerous consequences in a cell, such as the inactivation of enzymes by oxidation, alteration of the cell redox potential, oxidative damage of DNA resulting in mutations, changes in chromosome number and structure (Cassels and Curry, 2001).

The antioxidative system in plants, as one of the first defensive barriers, is set to sustain and extenuate the effects of ROS after the application of herbicides. Peroxidases (POD) and superoxide dismutases (SOD), together with glutamine synthetase (GS), catalases (CAT) and ascorbate peroxidases (APX), are the main components of the cell antioxidative system (Devi and Prasad, 2005). Peroxidases (EC 1.11.1.7) are encoded by a number of genes and are presented by a family of structurally similar isoforms with the same mechanism in plant cells. They catalyze the H_2O_2-dependent oxidation of a wide variety of reducing substrates, such as pyrogallol or guaiacol (Mittler, 2002). POD also has a role in very important physiological processes such as the control of growth by lignifications, cross-linking of pectins and structural proteins in the cell wall, seed germination and the catabolism of auxins (Prodanović et al., 2007). Superoxide dismutases (EC 1.15.1.1), together with catalases, are the most efficient antioxidative enzymes. As the first step in the removal of ROS

they catalyze the dismutation of superoxide to hydrogen peroxide, which is then scavenged by POD (Mittler, 2002).

The production of crops resistant to non-selective herbicides was one of the most important goals in agricultural research during the last decades. This resistance can be achieved using traditional breeding techniques (Damiani et al., 1990) or by direct gene transfer (Shu et al., 2005) and *Agrobacterium*-mediated transformation (Lohar et al., 2001; Trieu and Harrison, 1996; Kreig et al., 1990). The introduction of the *bar* gene coding for phosphinothricin acetyltransferase (PAT) provides plants with the ability to detoxify PPT and gives them resistance to this type of herbicide. This gene which was cloned from *Streptomyces hygroscopicus* and placed under the control of the 35S cauliflower mosaic virus promoter (35S CaMV) has been successfully transferred in legumes as well as other plants (Paz et al., 2004; Lohar et al., 2001; Trieu and Harrison, 1996).

Lotus corniculatus (*Fabaceae*) is one of the most popular commercially used pasture and forage legume crops in a number of regions worldwide. The presence of numerous weed species in *L. corniculatus* fields could be a major obstacle in commercial seed production. The herbicide Basta® is very often used for weed control and therefore obtaining PPT-resistant plants is very important. In this work we analyzed possible changes in activities of antioxidative enzymes (POD and SOD) in response to the application of Basta® herbicide in PPT-resistant *L. corniculatus* cv. Bokor plants derived by *Agrobacterium*-mediated transformation.

MATERIALS AND METHODS

Plant materials

Lotus corniculatus cv. Bokor, selected in the Center for Agriculture and Technological Research in Zaječar, Serbia, was used. Cotyledons from 10-day old seedlings were previously transformed in our laboratory with the *Agrobacterium tumefaciens* strain AGL1 carrying a binary vector pDM805 according to Tingay et al. (1997). This vector contained the *bar* gene coding for phosphinothricin acetyltransferase (PAT) and the *uidA* gene coding for the ß-glucoronidase (GUS) enzyme. Shoots were regenerated from cotyledonary explants (genotype 15) on selective media containing increasing PPT concentrations (5-7 mg l^{-1}) over twenty weeks. The well-grown PTT-resistant shoots, as well as the untransformed controls derived from the same genotype, were maintained on an MS nutrition medium (Murashige and Skoog, 1962), supplemented with 3% sucrose, 100 mg l^{-1} myo-inositol and solidified in 0.7% agar, pH 5.8 for three weeks. Culture conditions during the shoot elongation stages included a 16 h photoperiod at 25 ± 2°C. Transformation was confirmed by Southern blot analysis (data not shown).

In vitro Basta® resistance test

Putatively transformed and PTT-resistant, as well as control shoots, were tested with Basta®. The shoots were immersed for a few minutes in 300 mg l^{-1} herbicide solution. To make this solution, 20 µl of Basta® (the concentration of the active compound PPT in this solution was 150±9 g l^{-1}) was added to 10 ml of sterile water, which is equivalent to the doubled recommended field level of 5 kg h^{-1}. Screening for Basta® resistance (that included the measuring of fresh weights and shoot heights), was performed 6 days after herbicide application.

Protein extraction

Shoot tissues for POD and SOD analysis (0.5 g fresh weight) were collected 6 days after Basta® application and ground in liquid nitrogen. The tissue powder was transferred to an extraction buffer (100 mM K-phosphate buffer, pH 6.5, containing 1 mM phenylmethylsulfonyl fluoride and 1% polyvinylpyrolidone (w/v)), homogenized and allowed to thaw slowly at 4°C. The homogenate was then centrifuged for 15 min at 10000 g at 4°C. The soluble protein fraction was quantified by the Bradford method (1976) modified for Micro Plate Reader LKB 5060-007 (GDV, Roma, Italy). Absorbance was

measured at 620 nm and BSA was used as a standard.

POD and SOD assays

To visualize the POD isoforms, isoelectric focusing (IEF) was carried out in a 7.5% polyacrylamide gel (PAGE) with 3% ampholite in a pH gradient from 3 to 9. The gels were pre-focused for 30 min. Twenty five μg of the soluble protein fraction was loaded onto the gel. A constant voltage of 2000 V and an initial current of 50 mA were applied. After electrophoresis, the gels were immersed in 10% 4-chloro-α-naphthol and 3% H_2O_2 in 100 mM K- phosphate buffer (pH 6.5). Additionally, the POD activity was assayed in the crude extract (10 μl) in an incubation mixture containing 20 mM pyrogallol (A_{430} e = 2.47 $mM^{-1}cm^{-1}$), 4 mM H_2O_2, 100 mM K-phosphate buffer (pH 6.5) at 30°C. Enzyme activity was determined spectrophotometrically at 430 nm (Shimadzu UV - 160, Kyoto, Japan).

Native electrophoresis on 5% stacking and 10% running gels, with a reservoir buffer consisting of 0.025 M Tris and 0.192 M Glycine (pH 8.3), was performed at 24 mA for 120 min to determine SOD on the gels. The amount of total protein applied to each well was 20 μg. The superoxide dismutases were visualized after gel incubation in the dark in 50 ml of 0.1 M K-phosphate buffer (pH 7.8) containing 0.1 M EDTA, 0.098 mM nitroblue tetrazolium and 2 mM TEMED. After incubation for 30 min, the gel was rinsed in sterile water and left under UV light for 15 min, after which bands were visible.

The intensity of the POD and SOD bands was analyzed using TotalLab TL 120 software (Nonlinear Dynamics Ltd.) and the bands from non-treated control samples were used as standards for normalization.

Data analysis

Data are presented as the means ± standard errors, and were tested for statistical significance using analysis of variance (ANOVA) with the STATGRAPHICS Centurion XV ver.15.1.02 program. Values were considered significantly different when the probability (p) was less than 0.05. For the *in vitro* Basta® resistance test, twenty shots from each treatment were used. Analysis of enzyme activities was done in reps of three for each group.

RESULTS

In vitro Basta® resistance

Six days after the application of 300 mg l^{-1} PPT the control untransformed shoots had an obviously changed morphology, reduced growth and the leaves were affected with necrosis (Fig. 1). Shoot increment was significantly lower compared to the non-treated control shoots (Fig. 2A). The average increment of 20 shoots was 7.85 mm for herbicide-treated and 15.75 mm for non-treated samples. Measuring of the fresh shoot weights unexpectedly showed that the treated dying control shoots had very similar weights to all the other groups (Fig. 2B).

Transformation of *L. corniculatus* with the *bar* gene apparently led to the development of a resistant genotype whose shoots looked well and in good shape after the application of the herbicide in doses lethal for the control untransformed plants (Fig. 1A). Those shoots had an average increment of 20.25 mm, longer than both the non-treated transformed (12.70 mm) and non-treated untransformed shoots (15.75 mm) (Fig. 2A). The fresh shoot weights again did not significantly differ in the transformed shoots before and after Basta® application (Fig. 2B).

Antioxidative Enzymes Activity and Isoenzyme Pattern

The POD and SOD enzyme activities were analyzed in the control, untransformed *L. corniculatus* shoots, and in the *bar* gene carrying transformed shoots, before and after *in vitro* application of the non-selective herbicide Basta®.

Fig. 1. (A) Morphological changes on *in vitro* grown control shoots of *L. corniculatus* 6 days after application of the herbicide Basta® with PPT as an active compound, compared with non-treated control and herbicide-resistant treated or non-treated transgenic shoots. (B) Details from control and transformed PPT-resistant shoots 6 days after herbicide Basta® application.

Peroxidases

The POD specific activity and isoenzyme pattern showed changes in the control shoots treated with Basta® compared with the non-treated shoots, as well as in the transformed compared with the control untransformed shoots. After isoelectrofocusing, all seven different isoforms were present only in samples from the untransformed shoots after Basta® application (Fig. 3) which corresponds to the highest level of POD activity noticed in these samples (Fig. 4).

The specific activity of total soluble POD per total proteins in the control samples was 11.82 U mg^{-1} total proteins. After application of the herbicide this value increased to 16.74 U mg^{-1} total proteins and this was the highest measured activity. In the transformed shoots the activity of total POD was higher than in the untransformed shoots, but after application of Basta® this activity didn't change.

The POD isoenzyme pattern was qualitatively similar for basic forms in all the samples, but only the untransformed Basta® treated shoots had one additional acidic POD isoform. This isoform was with p*I* between 4.6 and 5.1. On the basic side of the gels six POD isoforms with p*I* values from 8.6 to 9.1 were detected. The most prominent POD isoform was the basic form with p*I* 8.6 (Fig. 3), present in all samples but with different levels of activity.

Superoxide dismutases

Application of the herbicide increased the activity of SOD both in the control and transformed *L. corniculatus* shoots grown *in vitro.* The analysis of the SOD bands derived by native PAGE showed the existence of only one enzyme form in all samples (Fig. 5A).

The normalized values of band intensity showed that the highest activity was in the control

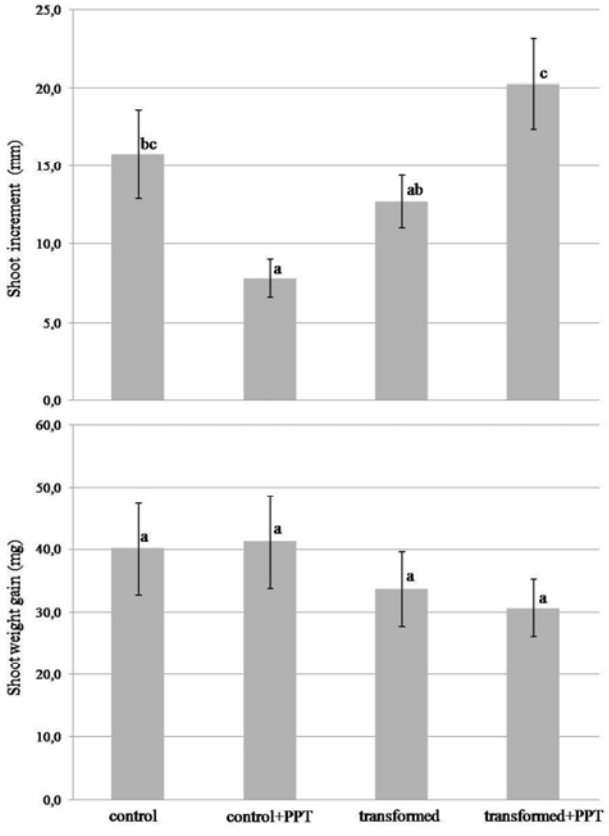

Fig. 2. Effect of PPT from Basta® treatment on growth of *L. corniculatus* control and transgenic PPT-resistant shoots under *in vitro* conditions 6 days after herbicide application. Y axis represented shoot increments or weight gains are expressed as mean±SE of 20 replicates. Values with the same letters are not significantly different at $p \leq 0{,}05$ level according to LSD test.

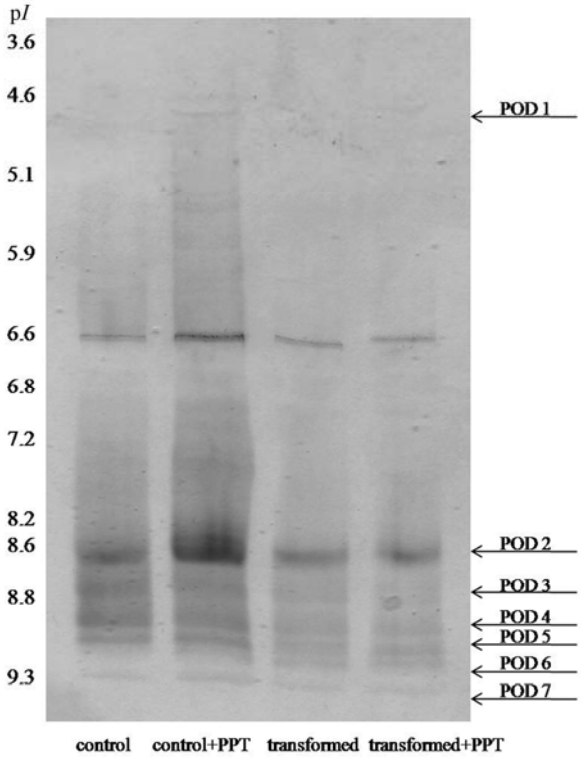

Fig. 3. IEF pattern for POD isoforms in control and transformed PPT-resistant *L. corniculatus* shoots, treated or not with PPT from Basta®. Arrows indicate POD isoforms with different p*I* values.

shoots treated with Basta®, but this increase was not as significant as we expected. The relative value for activity of SOD in this band was only 8.45% higher than in the band obtained from the control non-treated shoots with a relative value for activity of 100 (Fig. 5B). The transformed shoots showed lower levels of SOD activity than in the controls. The treatment with Basta® obviously had an influence on the *bar* gene carrying shoots. Surprisingly, six days after application of the herbicide the activity of SOD in the transformed shoots was almost doubled (42.27 before and 74.7 after treatment).

DISCUSSION

After *Agrobacterium*-mediated introduction of *bar* gene in *L. corniculatus* we obtained shoots with a high level of resistance to PTT types of herbicide (data not shown). In this work we tried to confirm the benefits obtained after this transformation and to show the capacity of these plants to reduce the oxidative stress caused after herbicide application.

Phosphinothricin (PTT), as an active ingredient of a wide spectrum of non-selective herbicides used in agriculture, effects some of the most important processes in plants soon after application (Evstigneeva et al. 2003). In plant cells, PPT and similar compounds affect the metabolic processes of nitrate assimilation and photorespiration which results in a

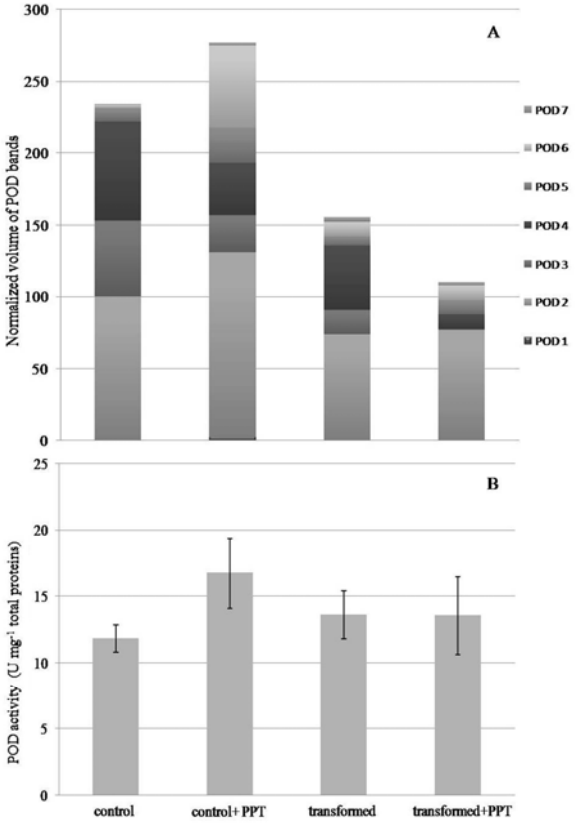

Fig. 4. Activity of total POD in control and transgenic PPT-resistant shoots of *L. corniculatus* grown under *in vitro* conditions:
A) derived from IEF gel using densitometrically based normalization (the most intensive band in the control non-treated sample was used for normalization);
B) obtained on spectrophotometer and expressed on total proteins determined with pyrogallol as the reducing substrate for peroxidase reaction. Each data bar represents the mean value of POD activity from three different samples of the same tissue type and variation is given as standard error (SE).

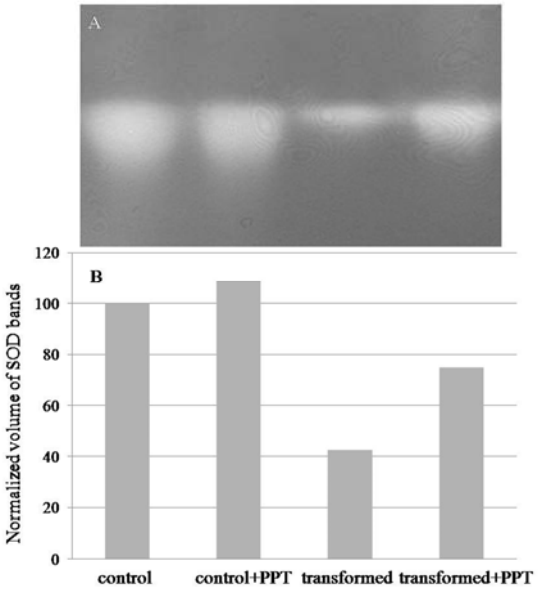

Fig. 5. The SOD activities in response to application of Basta® in *L. corniculatus* control and transgenic PPT-resistant shoots. Bands obtained after SOD specific *in gel* staining were normalized using TotlaLab TL120 software. The values are given as relative units; a control non-treated band was used as standard for normalization.

rapid increase in ammonium content, and subsequently a shift in amino acid spectrum (Krieg et al., 1990). Also, they can inhibit one of the most important enzymes in plants, RuBisCO, by the accumulation of glyoxylate and phosphoglycolate (Wild and Wendler, 1993). Qian et al. (2008) showed that only 12-96 h were enough to cause physiological changes in the aquatic unicellular alga, *Chlorella vulgaris,* after application of a herbicide with glufosinate as the active compound.

Induced transgenic *L. corniculatus* shoots grown *in vitro* did not show any differences in morphology when compared with the control untransformed shoots. Six days after application of the herbicide Basta®, the morphology of these shoots was still completely the same as that of the control non-treated shoots. The shoot increment and fresh weight gain were not different. However, untransformed PPT-sensitive shoots were significantly smaller, with leaves partially or fully affected by necrosis. This corresponds with the already described role of glufosinate ammonium in causing slight chlorosis and reduction of the chlorophyll content in *Oryza sativa* (Ahn, 2008). Although differences in shoot lengths were observed in the control and transformed shoots after herbicide application, the fresh weights were very similar for all groups. The PPT-sensitive treated shoots were notably smaller, with a reduced number of yellow leaves, but their average fresh weights were about the same as those

of the non-treated control or transformed resistant shoots. This points to the presence of not only an altered morphology but also an altered physiology and requires further investigation.

Herbicides cause oxidative damage in plants, either directly or indirectly by producing a high amount of ROS (Qian et al., 2008). Plant organisms have a suite of protective antioxidant enzymes and substances and the induction of this enzyme's activity often accompanies a disturbance of redox homeostasis and is used to indicate oxidative stress (Platiša et al., 2008). Very quickly after application, PPT or similar herbicides can cause a tremendous increase in the activity of these enzymes. The maximum activities of POD and SOD of about 2.9 times more than the control were measured in *C. vulgaris* four days after herbicide application (Qian et al., 2008).

We have shown that the spectrophotometrically measured activity of POD did not change in the transformed PPT-resistant shoots after application of Basta®. Phosphinothricin acetyltransferase (PAT), a product of the inserted *bar* gene, successfully eliminated PPT and did not allow it to be harmful and potentially stressful to treated shoots. The IEF isoform patterns were slightly changed after the herbicide treatment, but the number of detected isoforms, as well as the activity of the most pronounced bands, were very similar and completely matched with the activity measured on the spectrophotometer. Additionally, in the untransformed shoots, the application of Basta® provoked the increase of POD activity by about 42% and the induction of the new isoform. It is obvious that without PAT activity the application of PTT leads to a disturbance in some of the components of the antioxidative system in plants and the production of very harmful H_2O_2, which is a substrate for POD in cells.

Analysis of the SOD activity in our transformed shoots showed that insertion of the *bar* gene in the *L. corniculatus* genome and the presence of PAT did not completely reduce the oxidative stress caused by PPT treatment. An increase of almost 50% in band intensity suggests that the applied concentration of PPT is not lethal for the shoots but was sufficient to raise the level of SOD activity. In contrast, the activity of SOD only slightly increased after herbicide application to the untransformed shoots. This was unexpected, but we believe that an explanation for this lies in the fact that the cells in the untransformed herbicide-sensitive shoots six days after Basta® application in lethal concentrations, were already damaged and the amount of total active proteins was reduced compared with non-treated cells.

In summary, we conclude that the *Agrobacterium*-mediated introduction of the *bar* gene in the genome of *L. corniculatus* resulted in a derivation of PPT-resistant *in vitro*-grown shoots. These shoots showed a high level of resistance after the application of the non-selective herbicide Basta® with PPT as the active compound in comparison with the untransformed control shoots. POD and SOD as components of the antioxidative system of plants were affected by herbicide application and their activities were higher in the untransformed sensitive shoots than in the PPT-resistant *bar* carrying shoots.

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